Biliary tract external drainage protects against multiple organs injuries of severe acute pancreatitis rats

via Heme Oxygenase-1 upregulation

Jin-Long Wang^{1*}, MD; Ying Chen^{1*}, MD; Xiao-Qing Song¹, MD; Mei-Ling Lu¹, MD; Bing Zhao¹, MD; Li Ma¹,
MD; Er-Zhen Chen^{1#}, MD, PhD; En-Qiang Mao^{1#}, MD, PhD

Abstract

Objective: To investigate the effect of biliary tract external drainage (BTED) on severe acute pancreatitis (SAP) in rats and the relationship with heme oxygenase-1 (HO-1) pathway.

Methods: Thirty SD rats weighing 250-300g were randomly assigned into five groups (n = 6): *sham surgery (SS) group, SAP group, SAP+BTED group, SAP+ zinc protoporphyrin IX (ZnPP) group, SAP+BTED+ZnPP group.*

The SAP model was induced via retrograde injection of 4% sodium taurocholate (1mL/kg) into biliopancreatic duct through duodenal wall. BTED was performed by inserting a cannula into the bile duct of SAP rats. Tissue and blood samples were collected 24 hours after surgery. Pathological changes in organs were scored. The level of amylase, alanine transaminase (ALT), aspartate aminotransferase (AST), diamine oxidase (DAO) , lipopolysaccharide (LPS), myeloperoxidase (MPO) and ability to inhibit hydroxyl radical($\cdot\text{OH}$) in serum were measured. The expression of hemeoxygenase-1 (HO-1), tumor necrosis factor- α (TNF- α), and interleukin-6 (IL-6) in tissues were analyzed by RT- PCR and western-blot.

Results : Organs damage in SAP rats was significantly alleviated by BTED ($p < 0.05$). Compared to the SAP group, the serum level of amylase, ALT, AST, DAO, MPO, and LPS were significantly lower in the SAP+BTED group, and the ability to inhibit $\cdot\text{OH}$ was significantly higher ($p < 0.05$). The BETD treatment led to a significant reduction of TNF- α , IL-6 level and a significant increase of HO-1 level in tissues than in SAP rats ($p < 0.05$). ZnPP significantly inhibited all above mentioned changes.

Conclusions: BTED protected multiple organs against SAP related injuries via HO-1 upregulation.

Key Words: Severe acute pancreatitis; Biliary tract external drainage; Hemeoxygenase-1

Introduction

Acute pancreatitis (AP) is one of the most common seen acute abdominal diseases requiring emergency hospitalization all over the world [1]. Although Most of AP is mild, however, severe acute pancreatitis (SAP) develops in about 15–20% of patients with AP, characterized by acute onset, rapid change, local complications, early progressing multiple organ failure, high morbidity and mortality [2-5].

In the pathogenesis of SAP, the levels of the pro-inflammatory factors TNF- α and IL-6 increase in the ileum, which contributes to intestinal tissue injury. On the one hand, Kupffer cells in the liver is activated by TNF- α and IL-6 from intestinal tissue via the portal vein and produce pro-inflammatory TNF- α and IL-6, thus inducing early liver injury and the further release of pro-inflammatory factors from hepatocytes. On the other hand, gut-derived cytokines reach the lungs by permeating the mesenteric lymph vessels, the thoracic duct, the subclavian artery and the right atrium, resulting in ARDS [6-8]. Liver-derived cytokines enter gut via bile to establish the inflammatory loop of the gut-liver axis and enter the lungs via systemic circulation to activate monocytes/macrophages in the lungs to increase inflammatory cytokine production, leading to ARDS and increased intestinal and hepatic inflammation [9-12]. These processes constitute “gut-liver-lung” cycle that ultimately induces a cytokine cascade, resulting in multiple organ dysfunctions, which may lead to MODS and even death [13].

Hemeoxygenase-1 (HO-1), which is also referred to as heat shock protein-32, is a rate-limiting enzyme in heme catabolism to engender carbon monoxide, biliverdin, and ferrous iron [14, 15]. It protects cells by anti-inflammatory, anti-apoptotic and antioxidant activities under stress. HO-1 pathway has been drawing the attention from basic and clinical researchers for more than four decades [16, 17]. Previous studies showed that HO-1 can be strongly induced under several critical conditions, such as hemorrhagic shock, sepsis, and graft

pancreatitis [18-20], etc. Since HO-1 is considered as a protective factor in the scenario of stress, the targeted induction of HO-1 is emerging as an important therapeutic method for the protection against the development of inflammatory and oxidant activities [21, 22]. Particularly, pretreatment with HO-1-inducing agent in animal models of hemorrhagic shock exerted protective effects on multiple organs, including the liver, the kidney and the lungs [23-25]. In clinical practice, the organ function in patients with shock due to SAP was significantly improved when received biliary tract external drainage (BTED) either by endoscopic naso-biliary drainage, cholecystectomy or gallbladder percutaneous catheter drainage. The incidence of infection and morbidity of MODS were significantly decreased as well. Animal studies have also indicated that BTED can alleviate the injure of vital organs and improve the survival of rats with shock [26, 27]. Based on the above knowledge, we hypothesized that bilirubin might play a role of negative feedback in the formation of HO-1. We deduce that BTED may induce compensatory increase in HO-1 expression via interrupting the enterohepatic circulation of bilirubin. Since shock occurs more frequently among SAP patients, we hypothesized that BTED may exert a protective effect on SAP patients via the up-expression of HO-1 in vital organs. Therefore, this study was designed to explore the effect of BTED on HO-1 expression in these organs of rats subjected to SAP.

Materials and Methods

Animal model

Thirty adult male Sprague-Dawley rats (250-300 g) were purchased from Slac Laboratory Animal Corporation, Shanghai. The animals were housed and fed in accordance with the guidelines of the Committee on the Care and Use of Laboratory Animal Resources that were approved by the Shanghai Jiao Tong University School of Medicine Animal Care and Ethics Committee and were provided with free access to food and water. Before surgery, the rats were fasted overnight but were allowed access to water.

The rats were randomly divided into five groups (n=6): sham surgery (SS) group, SAP group, SAP+BTED

group, SAP+ zinc protoporphyrin IX (ZnPP) group, SAP+BTED+ZnPP group. Rats in the SS group were subjected to pentobarbital anesthesia, laparotomy and closure of abdomen only. SAP model rats were anaesthetized and ventral laparotomy, then 4% sodium taurocholate (1 ml/kg body weight) was retrograde injected into the pancreatic duct using a micro-injection pump at an even rate of 3 ml/h, and the atraumatic vascular clamp was removed 5 minutes later. Finally, the abdominal incisions were closed with sutures. All procedures were performed using a sterile technique. BTED model rats were performed bile duct cannulation and ligation under pentobarbital anesthesia[11]. A laparotomy was performed, and a catheter (inner diameter 0.4 mm; outer diameter 0.8 mm; length 20 cm) was inserted into the bile duct. The distal end of the bile duct was ligated, and the catheter was passed through the flank of the rat to avoid the permeation of bile into the gut and to facilitate external collection of the bile. Subsequently, the abdomen was closed, and the rats were housed in restraining cages with free access to food and water. Animals in the SAP+ZnPP group and SAP+BTED+ZnPP group were given ZnPP, a specific HO-1 blocker (30 mg/kg body weight), by intraperitoneal injection 24 hours prior to surgery. All rats received resuscitation with a single injection (40 ml/kg of body weight) of isotonic sodium chloride after surgical procedure, and each rat was housed in individual cage with free access to food and water. The rats in each group were sacrificed 24 h after surgery.

Reagents

Sodium taurocholate powder was purchased from Sigma Corporation (purity $\geq$ 97%, 101185852); zinc protoporphyrin IX (ZnPP) was purchased from TocrisBioscience, Britain ; TRIzol was purchased from Invitrogen Corporation, USA. RIPA buffer was purchased from Beyotime Institute of Biotechnology. The fluorescence quantitative RT-PCR kit was purchased from TOYOBO. The primers for HO-1, TNF- α , IL-6 and β -actin were synthesized by Invitrogen and the sequences of these primers are presented in Table 1.

Histomorphologic assessment

The liver, pancreas and intestinal tissues were harvested for morphological studies. The 10% buffered formalin-fixed sample was embedded in paraffin, sectioned at 4µm thickness, stained using haematoxylin-eosin and then observed under an optical microscope in a blinded manner by two independent pathologists. Three sections from each tissue were randomly selected for histopathology score. The standardized scoring system presented in Table 2 was utilized [28].

Assessment of amylase, ALT and AST

Blood samples were collected from the abdominal aorta, and centrifuged at 3500g for 15 minutes at 4°C. The resulted plasma was kept at -80°C until measurement. The level of serum amylase, ALT and AST was measured using a BECKMAN COULTER UniCel® DxC 800 Synchron® Clinical System auto-analyser.

Analysis of the LPS, DAO and MPO levels via ELISA, the ability to inhibit •OH via Hydroxyl Free

Radical assay kit

Serum was harvested. The expression levels of LPS, DAO and MPO were measured by using an ELISA Kit (Westang, China) according to the manufacturer's protocol. The ability to inhibit •OH was measured by using an Hydroxyl Free Radical assay kit (Nanjing Jiancheng, China) according to the manufacturer's protocol.

Assessment of HO-1, TNF-α and IL-6 expression

Total RNA was extracted from homogenized liver, pancreas and intestine samples using TRIzol. The concentration of the total RNA was determined via ultraviolet spectrophotometry. Aliquots of 2 µg of total RNA were used for cDNA synthesis. The cDNA was amplified via PCR using primers specific for HO-1, TNF-α and IL-6. β-actin were used as internal controls (The primer sequences are presented in Table 2).

The protein level of HO-1 in the pancreas was analyzed by Western blot. The total protein extraction from pancreatic tissue (50µg/sample) was separated via 10 % SDS-polyacrylamide gel electrophoresis (PAGE) and transferred to PVDF membranes. The membranes were incubated overnight at 4 °C in a primary antibody against

HO-1 (Abcam) or β -actin (Cell Signaling Technology). Primary antibodies were rabbit polyclonal to HO-1 (0.014 μ g/ml) and mouse monoclonal to β -actin (1 μ g/ml). Then, the membranes were incubated for 1 h with an Horseradish Peroxidase conjugated secondary antibody. The results were visualized using an ImageQuant LAS400 digital imaging system (GE, USA).

Statistics

All data were expressed as the means $\pm$ standard deviation (SD). Differences between the experimental groups were analyzed via one-way analysis of variance (ANOVA) and unpaired Student *t test* using SPSS16.0 software. And $p < 0.05$ was considered to be statistically significant difference.

RESULTS

Histomorphology

Liver. Compared to the SS group, the liver structure of the SAP group was irregular. The liver cells were denatured and putrescent. Hepatic cord disorder, liver cell edema and ballooning degeneration were detected. Marked inflammatory cell infiltration was detected in the portal area. All of these phenomena were attenuated in the SAP+BTED group. But in the SAP+ZnPP and SAP+BTED+ZnPP groups, these phenomena were exacerbated (Figure 1).

Pancreas. Pancreatic injury was more severe in the SAP and SAP+BTED groups than the SS group. Compared to the SAP group, the extent of gland damage, cell damage, neutrophil infiltration, hyperaemia and haemorrhage was mitigated in the SAP+BTED group. All of these phenomena were exacerbated in the SAP+ZnPP and SAP+BTED+ZnPP groups (Figure 1).

Intestine. Compared to the SS group, the epithelial cells of the small intestinal villi of the SAP group displayed necrosis and exfoliation, and inflammatory cell infiltration was detected. The phenomena of putrescence and desquamation of the epithelial cells in the intestinal mucosa were attenuated in the SAP+BTED group and were

exacerbated in the SAP+ZnPP and SAP+BTED+ZnPP groups (Figure 1).

Histopathologic scores

Compared to the SS group, histopathologic scores of liver, pancreas and intestine in the SAP group was significantly higher. Histopathologic scores in the SAP+BTED group decreased significantly compared with those in the SAP group. Histopathologic scores in the SAP+ZnPP group increased significantly compared with those in the SAP group. Histopathologic scores in the SAP+BTED+ZnPP group were significantly higher compared with those in the SAP and SAP+BTED groups, respectively. There was no significant differences found between the SAP+ZnPP and SAP+BTED+ZnPP groups concerning histopathologic scores. (Figure 1)

Levels of amylase, ALT, AST, LPS, DAO , MPO activity and the ability to inhibit •OH

The levels of AST, ALT and amylase activity in serum from the SAP group significantly increased compared to the SS group ($p<0.05$), and BTED treatment appeared to result in reduced activity levels compared to the SAP group ($p<0.05$). The levels of AST, ALT and amylase activity in serum from the SAP+BTED+ZnPP and SAP+ZnPP groups significantly increased compared to the SAP and SAP+BTED group, respectively ($p<0.05$) (Figure 2).

The levels of LPS, DAO and MPO in serum from the SAP group were higher than those in the SAP+BTED group ($p<0.05$), but were lower than those in the SAP+ZnPP group ($p<0.05$). The levels were found to be significantly lower in the SAP and SAP+BTED group than those in the SAP+BTED+ZnPP group ($p<0.05$). The ability to inhibit •OH in serum from the SAP+BTED group was higher than those in SAP, SAP+ZnPP and SAP+BTED+ZnPP group ($p<0.05$) (Figure2,5).

Effects of BTED on the expression of TNF- α and IL-6.

The mRNA transcription levels of TNF- α and IL-6 in the liver, the pancreas and the intestine in the SAP group significantly increased compared to the SS group ($p<0.05$). The mRNA expression levels of TNF- α and

IL-6 reduced significantly in the SAP+BTED group ($p<0.05$) and significantly increased in the SAP+ZnPP group compared with the SAP group. The mRNA levels of TNF- α and IL-6 in the SAP+BTED+ZnPP group significantly increased compared to the SAP and SAP+BTED groups ($p<0.05$) (Figure 3).

Effects of BTED on the expression of HO-1.

The expression levels of HO-1 in the intestine, liver, and pancreas in the SAP group significantly increased compared to the SS group ($p<0.05$). The expression levels of HO-1 in these tissues in SAP+BTED group were even higher than that in the SAP group ($p<0.05$). The expression levels of HO-1 in the intestine, the liver, and the pancreas in the SAP+ZnPP group significantly reduced compared to that in the SAP group ($p<0.05$). The expression levels of HO-1 in the intestine, liver and pancreas in the SAP+BTED+ZnPP group significantly reduced compared to the SAP and SAP+BTED groups ($p<0.05$)(Figure4).

DISCUSSION

SAP is a kind of systematic disease that is most usually characterized by cytokine activation, pancreatic necrosis, MODS and SIRS [29,30].At present, multiple organ dysfunction syndrome (MODS) is still the leading contributors to high mortality in SAP [31, 32].The gut is identified as a central organ in the pathogenesis of MODS [33-35]. The intestinal mucosal barrier was easily damaged in SAP due to its extremely sensitive to ischemia and hypoxia caused by hypoperfusion along with the progression of SAP. The increased intestinal permeability induces a derangement in gut barrier function, which results in bacterial and endotoxin translocation. Endotoxin is an important initiator in TNF- α , IL-6 and IL-1 β cascade from the excessively activated macrophages, monocytes, and neutrophils, which in turn lead to the remote organ injury during SAP. These factors are pro-inflammatory factors to SIRS, and MODS [36-39].

Under normal circumstances, there are a large number of Kupffer cells strategically located in liver. Kupffer cells are activated and proliferated upon the gut-derived cytokines and endotoxin drained via the portal vein. The

1 TNF- α and IL-6 produced by activated Kupffer cells induce early liver injury, initiate more synthesis and release
2
3 of additional pro-inflammatory mediators, which eventually induces the cytokines cascade in liver [40-42].
4
5
6 Hepatic-derived cytokines from liver via the venous route, activate monocytes/macrophages in the lung, which
7
8
9 in turn produced inflammatory cytokines leading to SIRS and ARDS [12]. Gut-derived bacteria, endotoxin, and
10
11
12 inflammatory cytokines contained in the mesenteric lymph fluid can also be drained to the lung, via mesenteric
13
14
15 lymph vessels, the thoracic duct, subclavian and right atrium, hence inducing ARDS [7,8,43-45].
16

17 Thus the inflammatory loop via blood/lymphatic circulation, which is named “gut-liver-lung axis”, is
18
19
20 established, and the axis would result in increasing intestinal, hepatic, and pulmonary inflammation promoting
21
22
23 tissue injury [9,46]. Our results showed that biliary tract external drainage can decrease the expression and
24
25
26 release of TNF- α and IL-6 in the intestine, liver, and pancreas and reduce the pathologic injury of the intestine,
27
28
29 liver, and pancreas as well. ZnPP could inhibit those phenomenon significantly.
30

31 HO-1 catalyzes heme to biliverdin, CO and iron. All three resulted products display anti-inflammatory
32
33
34 properties via the down-regulation of cytokines, such as TNF- α and NF- κ Bp65 sub unit[47-49], which are
35
36
37 initiating factor in SIRS and play a central role in the pathologic progression of SIRS[50]. By this mechanism,
38
39
40 HO-1 protects cells against oxidative stress and inflammation-induced injury. This phenomenon indirectly
41
42
43 indicates that BTED has inhibitory effect on the expression of inflammatory factors such as TNF- α and
44
45
46 NF- κ Bp65. It has been shown that HO-1 can be strongly induced under several critical conditions, such as
47
48
49 hemorrhagic shock, sepsis, and graft pancreatitis [20-22]. Compared with the SAP group, the expression of
50
51
52 HO-1 in SAP+BTED group was increased, and the expression of ALT, AST, Amylase, LPS and DAO in serum
53
54
55 were decreased, ZnPP could inhibit those phenomenon significantly. It demonstrated that BTED protected
56
57
58 pancreas, liver, and intestine against injury via HO-1 up-regulation. Nuclear factor erythroid 2-related factor 2
59
60
61 (Nrf2) signaling pathway is an important part of the cellular defense system. Under normal physiological
62
63
64
65

conditions, Nrf2 inhibited by Kelch-like ECH-associated protein 1 (Keap1) and stranded in the cytoplasm, and in oxidative stress were activated, released and transferred to the nucleus. Then gene transcription is initiated by the binding of the antioxidant response element in the target gene sequence, and ultimately through the upregulation of anti oxidative enzymes, such as HO-1, etc., to clear the level of reactive oxygen species (ROS) [51]. In our study, we measured the ability of hydroxyl radical inhibition in rats, which indirectly reflected the level of ROS. Besides, the MPO level can also reflect the body's oxidative stress. Although BTED can increase the expression of HO-1, but whether this effect is related to the activation of Nrf2 signaling pathway remains to be elucidated.

ZnPP was used to inhibit the HO-1 and should be intraperitoneally injected 24 hours prior to surgery, the dose is 30 mg/kg body weight. Accordingly, rats in the SAP+ZnPP and SAP+BTED+ZnPP groups were given ZnPP (30 mg/kg) via intraperitoneal injection 24 hours prior to surgery.

In summary, the results of this study showed that BTED disrupts the "live-gut-lung" cytokine cycle exerting protective effects on multiple organs vulnerable to SAP-mediated injury and activates the HO-1 pathway, which inhibits the expression of pro-inflammatory cytokines TNF- α and IL-6 in multiple organs. Further investigation is still needed to elucidate the underlying molecular mechanisms of BTED therapy.

CONCLUSIONS

It is showed in present study that the expression of TNF- α & IL-6 and histopathologic scores can be significantly decreased by BTED in the intestine, liver, and pancreas under SAP conditions. ZnPP significantly inhibited these effects. Our results further demonstrate that BTED alleviates multiple organs injury during SAP in vivo via HO-1 upregulation.

Authors' contributions

JLW and YC participated in the surgical procedure, the design of the protocol, drafted the manuscript, and performed the assay of HO-1 activity, ELISA, immunohistochemistry, Real-time PCR and the histologic analysis.

1 BZ and XQS performed the statistical analysis and helped to draft the manuscript. LM and MLL carried out the
2
3 Western blotting, and participated in the surgical procedure. EQM and EZC designed the study and helped to
4
5 draft the manuscript.
6
7

8 **Acknowledgements**

9
10
11 This study was supported by the National Science Foundation of China (NO. 81171789) , the Science and
12
13 Technology Committee of Shanghai (NO. 114119a4800) and the National Nature and Science Foundation for
14
15 Youth Scholar of China (No. 81501643).
16
17

18 **References:**

- 19
20
21
22 1 Yadav D, Lowenfels AB: The epidemiology of pancreatitis and pancreatic cancer. *Gastroenterology* 2013;
23
24 144:1252–1261.
25
26
- 27
28 2 Frossard JL, Steer ML, Pastor CM: Acute pancreatitis. *Lancet* 2008; 371: 143–152.
29
30
- 31
32 3 Vege SS, Gardner TB, Chari ST, Munukuti P, Pearson RK, Clain JE, Petersen BT, Baron TH, Farnell MB,
33
34 Sarr MG:Low mortality and high morbidity in severe acute pancreatitis without organ failure: a case for revising
35
36 the Atlanta classification to include ‘moderately severe acute pancreatitis’. *Am J Gastroenterol* 2009; 104:
37
38 710–715.
39
40
- 41
42 4 Andersson B, Appelgren B, Sjodin V, Ansari D, Nilsson J, Persson U, Tingstedt B, Andersson R: Acute
43
44 pancreatitis--costs for healthcare and loss of production. *Scand J Gastroenterol* 2013;48:1459-1465.
45
46
- 47
48 5 Banks PA, Bollen TL, Dervenis C, Gooszen HG, Johnson CD, Sarr MG, Tsiotos GG, Vege SS:
49
50 Classification of acute pancreatitis--2012: revision of the atlanta classification and definitions by international
51
52 consensus. *Gut* 2013;62:102-111.
53
54
- 55
56 6 Gonzalez RJ, Moore EE, Ciesla DJ, Biffl WL, Johnson JL, Silliman CC: Mesenteric lymph is responsible
57
58 for post-hemorrhagic shock systemic neutrophil priming. *J Trauma* 2001;51:1069-1072.
59
60
61
62
63
64
65

- Davidson MT, Deitch EA, Lu Q, Osband A, Feketeova E, Nemeth ZH, Hasko G, Xu DZ: A study of the biologic activity of trauma-hemorrhagic shock mesenteric lymph over time and the relative role of cytokines. *Surgery* 2004;136:32-41.
- Fanous MY, Phillips AJ, Windsor JA: Mesenteric lymph: the bridge to future management of critical illness. *JOP* 2007;8:374-399.
- Halpern MD, Holubec H, Dominguez JA, Meza YG, Williams CS, Ruth MC, McCuskey RS, Dvorak B: Hepatic inflammatory mediators contribute to intestinal damage in necrotizing enterocolitis. *Am J Physiol Gastrointest Liver Physiol* 2003;284:G695-G702.
- Towfigh S, Heisler T, Rigberg DA, Hines OJ, Chu J, McFadden DW, Chandler C: Intestinal ischemia and the gut-liver axis: an in vitro model. *J Surg Res* 2000;88:160-164.
- Jackson GD, Dai Y, Sewell WA: Bile mediates intestinal pathology in endotoxemia in rats. *Infect Immun* 2000;68:4714-4719.
- Callery MP, Kamei T, Mangino MJ, Flye MW: Organ interactions in sepsis. Host defense and the hepatic-pulmonary macrophage axis. *Arch Surg* 1991; 126:28-32.
- Gunjaca I, Zunic J, Gunjaca M, Kovac Z: Circulating cytokine levels in acute pancreatitis-model of sirs/cars can help in the clinical assessment of disease severity. *Inflammation* 2012; 35:758-763.
- Motterlini, R., and R. Foresti. 2014. Heme oxygenase-1 as a target for drug discovery. *Antioxidants and Redox Signaling* 20: 1810–1826.
- Ryter, S.W., and A.M. Choi. 2009. Heme oxygenase-1/carbon monoxide: from metabolism to molecular therapy. *American Journal of Respiratory Cell and Molecular Biology* 41: 251–260.
- Janssen A, Fiebiger S, Bros H, Hertwig L, Romero-Suarez S, Hamann I et al (2015) Treatment of chronic experimental autoimmune encephalomyelitis with epigallocatechin-3-gallate and glatiramer acetate alters

expression of heme-oxygenase-1. PLoS One 10:e0130251

17 Surolia R, Karki S, Kim H, Yu Z, Kulkarni T, Mirov SB et al (2015) Heme oxygenase-1 mediated autophagy protects against pulmonary endothelial cell death and development of emphysema in cadmium treated mice. Am J Physiol Lung Cell Mol Physiol. doi:10.1152/ajplung.00097

18 Takahashi T, Shimizu H, Morimatsu H, Maeshima K, Inoue K, Akagi R, Matsumi M, Katayama H, Morita K: Heme oxygenase-1 is an essential cytoprotective component in oxidative tissue injury induced by hemorrhagic shock. J Clin Biochem Nutr 2009; 44:28-40.

19 Takaki S, Takeyama N, Kajita Y, Yabuki T, Noguchi H, Miki Y, Inoue Y, Nakagawa T, Noguchi H: Beneficial effects of the heme oxygenase-1/carbon monoxide system in patients with severe sepsis/septic shock. Intensive Care Med 2010; 36:42-48.

20 Becker T, Zu VA, Terbish T, Klempnauer J, Jorns A: Induction of heme oxygenase-1 improves the survival of pancreas grafts by prevention of pancreatitis after transplantation. Transplantation 2007; 84:1644-1655.

21 Mai, J., Q. Qiu, Y.Q. Lin, N.S. Luo, H.F. Zhang, Z.Z. Wen, J.F. Wang, and C. YangXin. 2014. Angiotensin II-derived reactive oxygen species promote angiogenesis in human late endothelial progenitor cells through heme oxygenase-1 via ERK1/2 and AKT/PI3K pathways. Inflammation 37: 858–870.

22 Dey, S., C.M. Sayers, I.I. Verginadis, S.L. Lehman, Y. Cheng, G.J. Cerniglia, S.W. Tuttle, M.D. Feldman, P.J. Zhang, S.Y. Fuchs, et al. 2015. ATF4-dependent induction of heme oxygenase 1 prevents anoikis and promotes metastasis. The Journal of Clinical Investigation 125: 2592–2608.

23 Kubulus D, Mathes A, Pradarutti S, Raddatz A, Heiser J, Pavlidis D, Wolf B, Bauer I, Rensing H: Hemin arginate-induced heme oxygenase 1 expression improves liver microcirculation and mediates an anti-inflammatory cytokine response after hemorrhagic shock. Shock 2008; 29:583-590.

- 24 Arimori Y, Takahashi T, Nishie H, Inoue K, Shimizu H, Omori E, Kawanishi S, Toda Y, Morimatsu H,
Morita K: Role of heme oxygenase-1 in protection of the kidney after hemorrhagic shock. *Int J Mol Med* 2010;
26:27-32.
- 25 Maeshima K, Takahashi T, Uehara K, Shimizu H, Omori E, Yokoyama M, Tani T, Akagi R, Morita K:
Prevention of hemorrhagic shock-induced lung injury by heme arginate treatment in rats. *Biochem Pharmacol*
2005; 69:1667-1680.
- 26 Liu YJ, Mao EQ, Ouyang B, Chen J, Tang YQ, Huang SW, Guan XD: Effect of biliary tract external
drainage on cytokine expression and histomorphology of intestine, liver, and lung in rats with hemorrhagic shock.
Crit Care Med 2009; 37:2800-2806.
- 27 Jackson GD, Dai Y, Sewell WA: Bile mediates intestinal pathology in endotoxemia in rats. *Infect Immun*
2000; 68:4714-4719.
- 28 Schmidt J, Rattner DW, Lewandrowski K, Compton CC, Mandavilli U, Knoefel WT, Warshaw AL: A
better model of acute pancreatitis for evaluating therapy. *Ann Surg* 1992; 215:44-56.
- 29 Oiva J, Mustonen H, Kylanpaa ML, Kyhala L, Kuuliala K, Siitonen S, et al. Acute pancreatitis with organ
dysfunction associates with abnormal blood lymphocyte signaling: controlled laboratory study. *Crit Care*. 2010;
14: R207. doi: 10.1186/cc9329.
- 30 Mitchell RM, Byrne MF, Baillie J. Pancreatitis. *Lancet*. 2003; 361: 1447–1455.
- 31 Buter A, Imrie CW, Carter CR, Evans S, McKay CJ: Dynamic nature of early organ dysfunction
determines outcome in acute pancreatitis. *Br J Surg* 2002; 89:298-302.
- 32 Yasuda T, Ueda T, Takeyama Y, Shinzeki M, Sawa H, Nakajima T, Ajiki T, Fujino Y, Suzuki Y, Kuroda
Y: Significant increase of serum high-mobility group box chromosomal protein 1 levels in patients with severe
acute pancreatitis. *Pancreas* 2006; 33:359-363.

- 33 Leaphart CL, Tepas JR: The gut is a motor of organ system dysfunction. *Surgery* 2007; 141:563-569.
- 34 Sori AJ, Rush BJ, Lysz TW, Smith S, Machiedo GW: The gut as source of sepsis after hemorrhagic shock. *Am J Surg* 1988; 155:187-192.
- 35 Moore FA: The role of the gastrointestinal tract in postinjury multiple organ failure. *Am J Surg* 1999; 178:449-453.
- 36 Ammori BJ, Leeder PC, King RF, Barclay GR, Martin IG, Larvin M, McMahon MJ: Early increase in intestinal permeability in patients with severe acute pancreatitis: correlation with endotoxemia, organ failure, and mortality. *J Gastrointest Surg* 1999; 3:252-262.
- 37 Juvonen PO, Alhava EM, Takala JA: Gut permeability in patients with acute pancreatitis. *Scand J Gastroenterol* 2000;35:1314-1318.
- 38 McNaught CE, Woodcock NP, Mitchell CJ, Rowley G, Johnstone D, MacFie J: Gastric colonisation, intestinal permeability and septic morbidity in acute pancreatitis. *Pancreatology* 2002;2:463-468.
- 39 Rahman SH, Ammori BJ, Larvin M, McMahon MJ: Increased nitric oxide excretion in patients with severe acute pancreatitis: evidence of an endotoxin mediated inflammatory response? *Gut* 2003;52:270-274.
- 40 Harte AL, da Silva NF, Creely SJ, McGee KC, Billyard T, Youssef-Elabd EM et al. Elevated endotoxin levels in non-alcoholic fatty liver disease. *J Inflamm (Lond)* 2010; 7: 15.
- 41 Brundage SI, Schreiber MA, Holcomb JB, Zautke N, Mastrangelo MA, Xq X, Macaitis J, Tweardy DJ: Amplification of the proinflammatory transcription factor cascade increases with severity of uncontrolled hemorrhage in swine. *J Surg Res* 2003;113:74-80.
- 42 Martin C, Boisson C, Haccoun M, Thomachot L, Mege JL: Patterns of cytokine evolution (tumor necrosis factor-alpha and interleukin-6) after septic shock, hemorrhagic shock, and severe trauma. *Crit Care Med* 1997;25:1813-1819.

- 43 Xu DZ, Lu Q, Adams CA, Issekutz AC, Deitch EA: Trauma-hemorrhagic shock-induced up-regulation of endothelial cell adhesion molecules is blunted by mesenteric lymph duct ligation. Crit Care Med 2004;32:760-765.
- 44 Gonzalez RJ, Moore EE, Ciesla DJ, Biffl WL, Johnson JL, Silliman CC: Mesenteric lymph is responsible for post-hemorrhagic shock systemic neutrophil priming. J Trauma 2001;51:1069-1072.
- 45 Dayal SD, Hasko G, Lu Q, Xu DZ, Caruso JM, Sambol JT, Deitch EA: Trauma/hemorrhagic shock mesenteric lymph upregulates adhesion molecule expression and il-6 production in human umbilical vein endothelial cells. Shock 2002;17:491-495.
- 46 Yilin Wang , Weiyan Liu ,Xiqiang Liu ,Meng Sheng , Yaofei Pei ,Ruoqing Lei ,Shengdao Zhang , Ran Tao: Role of Liver in Modulating the Release of Inflammatory Cytokines Involved in Lung and Multiple Organ Dysfunction in Severe Acute Pancreatitis. Cell Biochem Biophys (2015) 71:765–776.
- 47 Zhou H, Qian H, Liu J, Zhu D, Ding W, Pan P, Jin D, Wang J, Li W: Protection against lung graft injury from brain-dead donors with carbon monoxide, biliverdin, or both. J Heart Lung Transplant 2011;30:460-466.
- 48 Sass G, Seyfried S, Parreira SM, Yamashita K, Kaczmarek E, Neuheuer WL, Tiegs G: Cooperative effect of biliverdin and carbon monoxide on survival of mice in immune-mediated liver injury. Hepatology 2004;40:1128-1135.
- 49 Kadl A, Pontiller J, Exner M, Leitinger N: Single bolus injection of bilirubin improves the clinical outcome in a mouse model of endotoxemia. Shock 2007;28:582-588.
- 50 Tracey KJ, Cerami A: Tumor necrosis factor: a pleiotropic cytokine and therapeutic target. Annu Rev Med 1994;45:491-503.
- 51 Kensler TW, Wakabayashi N, Biswal S. Cell survival responses to environmental stresses via the Keap1-Nrf2-ARE pathway[J]. Annu Rev Pharmacol Toxicol, 2007, 47: 89-116.

Figure legend

Figure1. Histopathologic changes and scores of the liver, the pancreas, the intestine. Images of sections stained with hematoxylin and eosin from the the liver, pancreas and intestine tissues of rats 24 h after the induction of severe acute pancreatitis or control treatment. Results are presented as means $\pm$ standard error ($n=6$). ^a $P < 0.05$ compared with the SS group; ^b $P < 0.05$ compared with the SAP group; ^c $P < 0.05$ compared with the SAP+BTED group (magnification, $\times 100$).

Figure2. Effect of biliary tract external drainage on indicators of organ functions. Data are presented as means $\pm$ SD($n=6$ for each group). ^a $P < 0.05$ compared with the SS group; ^b $P < 0.05$ compared with the SAP group; ^c $P < 0.05$ compared with the SAP+BTED group. Levels of amylase, ALT, AST, LPS and DAO activity decreased significantly after BTED under SAP conditions. ZnPP inhibited this effect.

Figure3. Gene expression of TNF- α and IL-6 in the liver, pancreas and intestine tissues of rats. Quantitative RT-PCR was used to assess the gene expression of TNF- α and IL-6 for rats in the following groups: SS group, SAP group, SAP+BTED group, SAP+ZnPP group, SAP+BTED+ZnPP group. A, B=liver; C, D=pancreas; E, F=intestine. ^a $P < 0.05$ compared with the SS group; ^b $P < 0.05$ compared with the SAP group; ^c $P < 0.05$ compared with the SAP+BTED group. The mRNA expression levels of TNF- α and IL-6 decreased significantly after BTED under SAP conditions. ZnPP inhibited this effect.

Figure4. The expression levels of HO-1 in the liver, pancreas and intestine tissues of rats. A, B and C are gene expression levels, D is protein expression level. A=liver; B=pancreas; C=intestine. ^a $P < 0.05$ compared with the SS group; ^b $P < 0.05$ compared with the SAP group; ^c $P < 0.05$ compared with the SAP+BTED group. The mRNA expression levels of HO-1 increased significantly after BTED under SAP conditions. ZnPP inhibited this effect.

Figure5. The level of MPO and the ability to inhibit $\bullet$ OH in serum. ^a $P < 0.05$ compared with the SS group; ^b P

1 < 0.05 compared with the SAP group; ^c $P < 0.05$ compared with the SAP+BTED group. The level of MPO
2
3 decreased significantly after BTED under SAP conditions, and the ability to inhibit •OH increased significantly.
4
5
6 ZnPP inhibited this effect.
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

Table 1. PCR primer sequences

Gene	Forward primer (5'-3')	Reverse primer (5'-3')
β-actin	TGG TGG GTA TGG GTC AGA AG	GAC AAT GCC GTG TTC AAT GG
HO-1	ACC CCA CCA AGT TCA AAC AG	GAG CAG GAA GGC GGT CTT AG
TNF-α	CCC AAT CTG TGT CCT TCT AAC T	CAC TAC TTC AGC GTC TCG TGT
IL-6	AGA GAC TTC CAG CCA GTT GC	AGC CTC CGA CTT GTG AAG TG

Table 2. Histopathologic scoring criteria**Oedema**

0	Absent
0.5	Focal expansion of interlobar septae
1	Diffuse expansion of interlobar septae
1.5	Same as 1 +focal expansion of interlobular septae
2	Same as 1 +diffuse expansion of interlobular septae
2.5	Same as 2 +focal expansion of interacinar septae
3	Same as 2 +diffuse expansion of interacinar septae
3.5	Same as 3 +focal expansion of intercellular space
4	Same as 3 +diffuse expansion of intercellular space

Acinar necrosis

0	Absent
0.5	Focal occurrence of 1-4 necrotic cells/HPF
1	Diffuse occurrence of 1-4 necrotic cells/HPF
1.5	Same as 1 +focal occurrence of 5-10 necrotic cells/HPF
2	Diffuse occurrence of 5-10 necrotic cells/HPF
2.5	Same as 2 +focal occurrence of 11-16 necrotic cells/HPF
3	Diffuse occurrence of 11-16 necrotic cells/HPF (foci of confluent necrosis)
3.5	Same as 3 +focal occurrence of >16 necrotic cells/HPF
4	>16 necrotic cells/HPF (extensive confluent necrosis)

Haemorrhage and fat necrosis

0	Absent
0.5	1 focus
1	2 foci
1.5	3 foci
2	4 foci
2.5	5 foci
3	6 foci
3.5	7 foci
4	≥8 foci

Inflammation and perivascular infiltrate

0	Absent
0.5	0-1 intralobular or perivascular leukocytes/HPF
1	2-5 intralobular or perivascular leukocytes/HPF
1.5	6-10 intralobular or perivascular leukocytes/HPF
2	11-15 intralobular or perivascular leukocytes/HPF
2.5	16-20 intralobular or perivascular leukocytes/HPF
3	21-25 intralobular or perivascular leukocytes/HPF
3.5	>30 leukocytes/HPF or focal microabscesses
4	>35 leukocytes/HPF or confluent microabscesses

HPF, high-power field.

Figure.1

Figure2

Figure3

Figure4

Figure5